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Evaluation of the biological toxicity of fluorine in Antarctic krill

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Abstract Antarctic krill is a potentially nutritious food source for humans, but fluorine (F) toxicity is a matter of concern. To evaluate the toxicity of F in Antarctic krill, 30 Wistar rats were divided into three groups with different dietary regimens: a control group, a krill treatment group (150 mg·kg^{-1} F), and a sodium fluoride (NaF) treatment group (150 mg·kg^{-1} F). After three months, F concentrations in feces, plasma, and bone were determined, and the degree of dental and skeletal fluorosis was assessed. The F concentrations in plasma and bone from the krill treatment group were 0.167 0±0.020 4 mg·L^{-1} and 2 709.8±301.9 mg·kg^{-1}, respectively, compared with 0.043 8±0.005 5 mg·L^{-1} and 442.4±60.7 mg·kg^{-1}, respectively, in samples from the control group. Concentrations of F in plasma and bone in the krill treatment group were higher than in the control group, but lower than in the NaF treatment group. The degree of dental fluorosis in the krill treatment group was moderate, compared with severe in the NaF treatment group and normal in the control group. The degree of skeletal fluorosis did not change significantly in any group. These results showed that the toxicity of F in Antarctic krill was lower than for an equivalent concentration of F in NaF, but it was toxic for rats consuming krill in large quantities. To conclude, we discuss possible reasons for the reduced toxicity of F in Antarctic krill. The present study provides a direct toxicological reference for the consideration of Antarctic krill for human consumption.

Keywords **Antarctic krill, fluorine (F), fluorosis, rat experiment, toxicological effect**

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1 Introduction

Antarctic krill (*Euphausia superba*) is one of the most abundant animal species in the Southern Ocean and it plays a pivotal role in Antarctic marine ecosystems. The mean total abundance of krill is about 7.8×10^{14}, and the gross post-larval production is 342–536 Mt·a^{-1}[1]. The total allowable catch for the krill fishery is about 5 Mt·a^{-1}, which could potentially result in a small reduction of the krill stock[2].

Antarctic krill is rich in nutrients and low in calories compared with traditional animal foods[3]. The protein recovery yield is 45%–50% (dry weight, DW), and the content of nine essential amino acids accounts for approximately 50% of the total amino acid content in Antarctic krill[4-5]. The concentrations of these nine essential amino acids are sufficiently high to meet the FAO/WHO/UNU criteria for

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human consumption[5]. It has been reported that the biological value of protein in krill is higher than for other meat proteins and milk proteins[6]. The lipid content of Antarctic krill ranges from 12%–50% on a DW basis, with high levels of omega-3 polyunsaturated fatty acids (ω-3 PUFAs). In particular, eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3) have been linked to a reduced risk of cardiovascular disease[7].

A variety of krill products including seafood, pharmaceuticals, chemical compounds, and animal feed have been produced[8]. With the increasing human population, a shortage of food may become a serious problem in the future. A large stock of Antarctic krill could become available for human consumption and Antarctic krill is potentially one of the most promising sources of energy and nutrients. Previous research has suggested that Antarctic krill could be one of the most valuable remaining marine resources[9]. However, krill has not been widely used for human consumption because of the high fluorine (F) content[10]. The total F concentration in the exoskeleton of krill is 3 828–4 278 $mg \cdot kg^{-1}$ DW, 178–285 $mg \cdot kg^{-1}$ DW in muscle, and 1 102–1 432 $mg \cdot kg^{-1}$ DW in whole krill. When krill are stored after being harvested, the F in the exoskeleton can transfer rapidly to the muscle[11].

Fluorine is commonly added to toothpaste or mouthwash to prevent dental caries[12], however, F may be harmful to human health, causing dental fluorosis and skeletal fluorosis when intake is excessive[13]. According to the recommended dietary allowance (2012), the intake of F should be less than 4 $mg \cdot d^{-1}$. Because the concentration of total F in whole krill is about 1 500 $mg \cdot kg^{-1}$, humans could safely ingest only 2.7 $g \cdot d^{-1}$ DW. If the Antarctic krill resource is to be used as a food for humans, the high concentration of F in krill needs to be addressed. In yellowtail (*Seriola quinqueradiata*) fed with 100% Antarctic krill meal, the F concentration in vertebral bone was 33 000 $mg \cdot kg^{-1}$. No histopathological changes were detected in liver tissue, and the yellowtail did not show any adverse effects[14]. However, there have been few studies conducted to directly determine whether F in Antarctic krill is toxic to rats or humans.

The objective of the present study was to assess the toxicity of F in whole Antarctic krill compared with F in NaF. We conducted an animal experiment to determine bone and plasma F concentrations and to assess dental fluorosis and skeletal fluorosis in Wistar rats fed a basal diet, a basal diet with added Antarctic krill powder, or a basal diet with added NaF. The present study provides valuable background data for the investigation of krill as a potential human food source.

2 Materials and Methods

2.1 Chemicals

Sodium fluoride (NaF) was obtained from the Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). The Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) provided the sodium hydroxide (NaOH), concentrated hydrochloric acid (HCl), sodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$), and phenol red ($C_{19}H_4O_5S$). All chemicals used in this study were analytical grade or above. The Shanghai Leici Instrument Factory (Shanghai, China) supplied an ionometer (PXSJ-226) and a fluorine ion selective electrode (FISE). The detection limit of the FISE was 10-6-10-1 $mol \cdot L^{-1}$. Ultra-pure water produced using a Millipore Milli-Q purification system (Milford, MA, USA) was used for the preparation of all chemical reagents.

2.2 Experimental animals and treatment

Thirty newly weaned Wistar rats were obtained from the Shanghai Laboratory Animal Center Co. Ltd. (Shanghai, China). The rats were kept under temperature-controlled (23±2℃), well-ventilated, and hygienic conditions with a 12 h dark/light cycle. They had ad libitum access to food and distilled water. After a one-week settling-in period, the rats were randomly assigned to one of three groups with 10 rats in each group. Rats in control group were fed with a basal diet obtained from the Shuangshi Laboratory Animal Feed Science Co. Ltd. (Suzhou, China). The basal diet contained 26% corn, 24.7% soybean meal, 34% flour, 5% fishmeal, 2.3% plant oil, 3% alfalfa meal, and 5% trace mineral premix. Rats in the NaF treatment group were fed the basal diet with added NaF. Rats in the krill treatment group were fed the basal diet mixed with Antarctic krill powder. The LD50 of F is 1 500 $mg \cdot kg^{-1}$ and one tenth of the LD50 is considered to be a high dose in an experimental setting. Therefore, the target F concentration in feed for the NaF and krill groups was about 150 $mg \cdot kg^{-1}$. For the krill group, freeze-dried Antarctic krill supplied by Keruier Biological Products Co. Ltd. (Shandong, China) was ground into powder. Both the NaF and krill powder were thoroughly mixed with the basal diet before feeding. The weight of food eaten was recorded daily, and the rats were weighed every week. After three months, the rats were sacrificed and specimens of bone and plasma were collected. This experiment was conducted in compliance with the Animal Use Protocol approved by the Institutional Animal Care and Use Committee of Soochow University (SYXK2007-0035).

2.3 Chemical analysis of F

2.3.1 Concentrations of acid extractable F in feed and feces

Samples of feed in powdered form were collected before the experiment and the F concentration was determined for the preparation of the experimental diet for the NaF and krill groups (150 $mg \cdot kg^{-1}$). The F concentration in the feed was also monitored in the middle and at the end of the experiment. At the end of the experiment, fresh fecal samples were collected, oven-dried at 50℃ for 24 h, ground, and kept in airtight plastic bags. The feed and fecal samples were extracted in 1 M hydrochloric acid (HCl), and F concentrations were determined using a FISE according to

the GB-T 13083-2002 standard method[15]. The extraction was carried out according to the following standard procedure. One gram of fresh pulverized feed or oven-dried feces was dissolved in 10 mL of 1 M HCl in a plastic container, then placed on a shaker at 150 rpm for 1 h at 25℃. Next, 25 mL of Total Ion Strength Adjustment Buffer (TISAB) solution were added to the container, and the solution was further diluted to 100 mL using ultra-pure deionized water. The extraction solution was filtered into a 250 mL plastic beaker for F concentration analysis. The TISAB solution was prepared by mixing 3 M sodium citrate solution and 0.75 M sodium acetate trihydrate solution (1/1, v/v).

2.3.2 Concentration of F in rat plasma

The rats were anesthetized using ether, and blood samples were collected from the inferior vena cava using heparinized tubes. The plasma was separated by centrifuging (3 000 rpm, 10 min) at 4℃, and stored at −20℃. The F concentration in plasma was determined using a FISE according to the WS-T 212-2001 method[16]. In brief, 0.5 mL of the plasma sample were measured into a 10 mL beaker, and then the TISAB solution was added to the same volume as the plasma sample. The solution was mixed using a magnetic mixer for 5 min prior to the FISE measurement. The TISAB solution was prepared as follows: 58.0 g of sodium chloride and 0.4 g of sodium citrate dehydrate were dissolved in 500 mL of ultra-pure deionized water, and then 57 mL of acetic acid were added to the solution. The solution pH was adjusted to 7 using 5 M sodium hydroxide, and the final solution was diluted to 1 000 mL with ultra-pure water.

2.3.3 Concentration of total F in rat bone

Rat femora were boiled in water to remove muscle tissue. The bone samples were oven-dried at 105℃ for 12 h, and then powdered in a mechanical agate mortar. The ground samples were kept in airtight plastic bags at 4℃ until chemical analysis. The total F concentration was determined using a FISE as described by Xie and Sun[17] and Yin et al.[18], with minor modifications. Briefly, 50 mg of dried pulverized bone were placed between two layers of sodium hydroxide (NaOH) in a Ni crucible and then heated at 318℃ for 30 min. The samples were then heated at 600℃ in a muffle furnace for 10 min. The residue was dissolved in water (about 10 mL) on a hot plate, and then transferred into 100 mL volumetric plastic bottles. Two drops of 0.1% phenol red were added to each bottle, and the solution pH was adjusted to a yellow color using 6 M HCl, and then adjusted to a light red color using 0.5% NaOH (w/v). Next, 20 mL of citrate sodium buffer were added, and the final solution was diluted to 100 mL using ultra-pure water. Finally, the concentration of total F in the solutions was determined using a FISE.

2.3.4 Quality assurance (QA) and quality control (QC)

The QC samples in this study included spiked samples, blanks, and the standard reference materials (GSS-2, GSS-5, Chinese National Institute of Minerals). The recovery rates of F in the spiked QC samples (including feed, feces, and plasma) and the standard reference materials ranged from 90%–110%. The precision of the F measurement was <5%. Concentrations of F in feed, feces, and plasma samples were determined along with the QC samples for quality control in this study.

2.4 Assessing dental and skeletal fluorosis

Dental fluorosis and skeletal fluorosis are two specific indictors of F toxicity[19]. To assess the toxicity of F in Antarctic krill, one rat from each group was randomly selected and photographs of the teeth were taken every 30 days using a Nikon D90 camera. The Dean's Index[20] was used to measure dental fluorosis. The index has six categories and the criteria for each category are as follows: (1) normal: enamel is smooth and uniform in color; (2) questionable: enamel may exhibit some white flecks or small white spots; (3) very mild: less than 25% of the tooth surface displays irregular white areas; (4) mild: more than 25% of the tooth surface but less than 50% is affected; (5) moderate: generalized areas of hypocalcification on all surfaces of the tooth, may exhibit attrition on susceptible tooth surfaces, and brown spots may be present; (6) severe: widespread brown stains and pitting.

The right femur of each rat was collected, immediately placed in 0.9% saline solution (w/v), and stored at 4℃ until analysis. The cortex of the distal femur was scanned using micro-computed tomography (μCT)[21-22] to assess the 3-D bone microstructure. In brief, the femora were placed on a carbon fiber holder during the course of the scan using a bench-top μCT system. The scan was performed with an 18-μm voxel size (Skyscan1176, Micro Technology Hong Kong Ltd., Belgium). The images were reconstructed and calibrated with the standard material for rat bone mineral density (BMD). Each final 3-D image was analyzed for BMD and other parameters using a CT-Analyzer. Each femur was cropped out of the reconstructed volume and rotated so the shaft axis coincided with the x-axis. The region of interest (i.e., cancellous bone and cortical bone) was then defined in each femur 3-D image. The following parameters were determined: BMD (kg·mm^{-3}), bone volume (BV, mm^3), tissue volume (TV, mm^3), bone surface area (BS, mm^2), tissue surface area (TS, mm^2), intersection surface area (IS, mm^2), bone surface area/bone volume ratio (BS/BV, mm^{-1}), bone surface density (BS/TV, mm^{-1}), mean trabecular thickness (TbTh, μm), trabecular number (TbN, mm^{-1}), and mean trabecular separation (TbSp, μm).

2.5 Statistical analysis

Statistical analysis was carried out using SPSS (version 19.0). The variation effects were determined by one-way ANOVA, and the level of significance (α) was set at 0.05. Data are presented as mean±standard deviation for the

control and each treatment group.

3 Results and discussion

All rats in the three groups were in good mental and physical health. There were no significant differences in body weight or food intake among the three groups (Data not shown).

3.1 Concentrations of HCl extractable F in feed and feces

Concentrations of HCl extractable F in feed and feces are shown in Figure 1. The concentrations of HCl extractable F in the powdered feed were 144.3±3.2 mg·kg^{-1} and 146.9±8.0 mg·kg^{-1} in the NaF and krill groups, respectively. The acid-extractable F concentration in the basal feed fed to the control group was 30.3±1.0 mg·kg^{-1}. The concentrations of HCl extractable F in the feces were 482.2±3.4 mg·kg^{-1} and 484.0±2.0 mg·kg^{-1} for the NaF and krill groups, respectively, and 70.2±4.0 mg·kg^{-1} in the control group. The acid-extractable F concentrations in feed and feces did not differ between the two treatment groups, indicating that the metabolism of F was similar for the two different sources of F (NaF and krill) and the absorption of F through the gastrointestinal tract was similar in the two treatment groups.

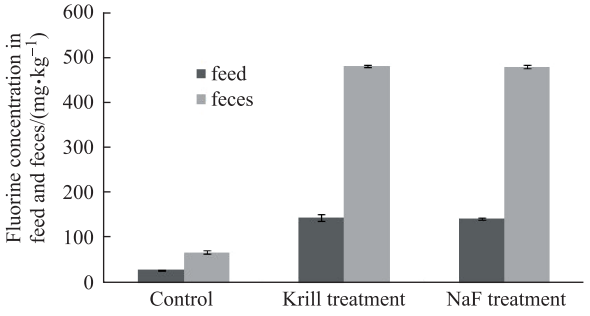

Figure 1 Concentration of F in the feed and feces of the control, krill treatment, and NaF treatment groups.

3.2 Concentration of inorganic F in plasma and total F in bones

Figure 2 shows the levels of inorganic F in plasma for the three groups. The F concentration in plasma from the krill treatment group was 0.167 0±0.020 4 mg·L^{-1}, higher than in the control group (0.043 8±0.005 5 mg·L^{-1}), but lower than in the NaF treatment group (0.196 4±0.026 0 mg·L^{-1}). Concentrations of inorganic F in the plasma from the NaF and krill treatment groups were significantly higher than the concentration in plasma from the control group (P<0.05). However, the concentration of inorganic F in the plasma from the krill treatment group was slightly lower than in plasma from the NaF group (P=0.065).

Concentrations of total F in femur bone are presented in Figure 3. The levels of total F in femur bone were 2 709.8±301.9 mg·kg^{-1} and 3 197.4±171.2 mg·kg^{-1} in the krill and NaF treatment groups, respectively. The level of total F in femur bone in the control group was 442.4±60.7 mg·kg^{-1}, significantly lower than in the two treatment groups (P<0.05). Femur bone from the krill treatment group had a lower F concentration than bone from the NaF treatment (P<0.05), but a significantly higher concentration than bone from the control group (P<0.05).

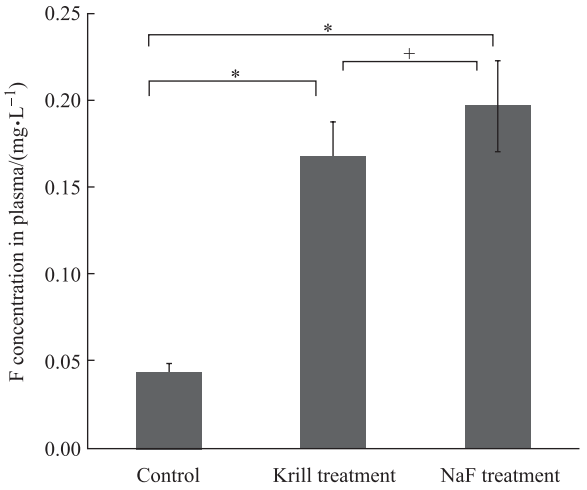

Figure 2 Concentration of F in the plasma of the control, krill treatment, and NaF treatment groups ($^{*}P$<0.05, ^{+}P>0.05).

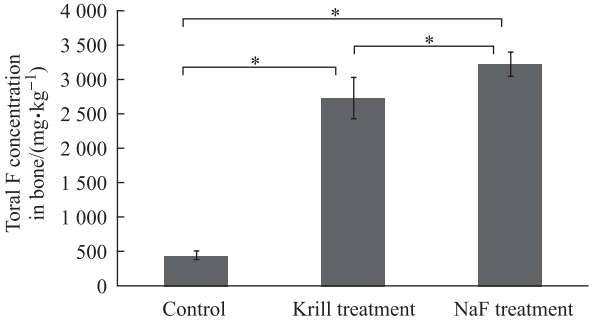

Figure 3 Total F concentration in the bone of the control, krill treatment, and NaF treatment groups ($^{*}P$<0.05, ^{+}P>0.05).

The plasma F level has been used as a biomarker to measure F exposure[23]. The F content in plasma depends on the dosage, time, and duration of exposure[24]. Dosage, time, and duration of exposure were similar in the NaF and krill treatment groups in this study. However, the plasma F concentration in the NaF treatment group was higher than in the krill treatment group. Absorbed F is rapidly distributed to the intracellular and extracellular fluids by the circulation, and is only retained in calcified tissues. The concentration of F in plasma can therefore be used as biomarker of acute exposure to F, and the concentration in bone can be used to quantify long-term exposure[25].

The high F concentrations in the plasma and bone tissue of rats in the krill treatment group could result in potential health risks. However, the F in krill may be less toxic to the rats compared with the F in NaF, because lower F concentrations in plasma and bone were observed in the krill treatment group during the present study.

3.3 Assessing dental and skeletal fluorosis

Photographs of the teeth of one rat from the control group and one rat from each of the two treatment groups were taken every 30 days during the study and are shown in Figure 4. The teeth of the rat in the krill group were classified according to Dean's Index as no fluorosis on day 30, very mild fluorosis on day 60, and moderate fluorosis on day 90. The classification of teeth from the rat in the NaF treatment group suggested greater F toxicity than in the krill treatment group, showing very mild fluorosis on day 30, moderate fluorosis on day 60, and severe fluorosis on day 90.

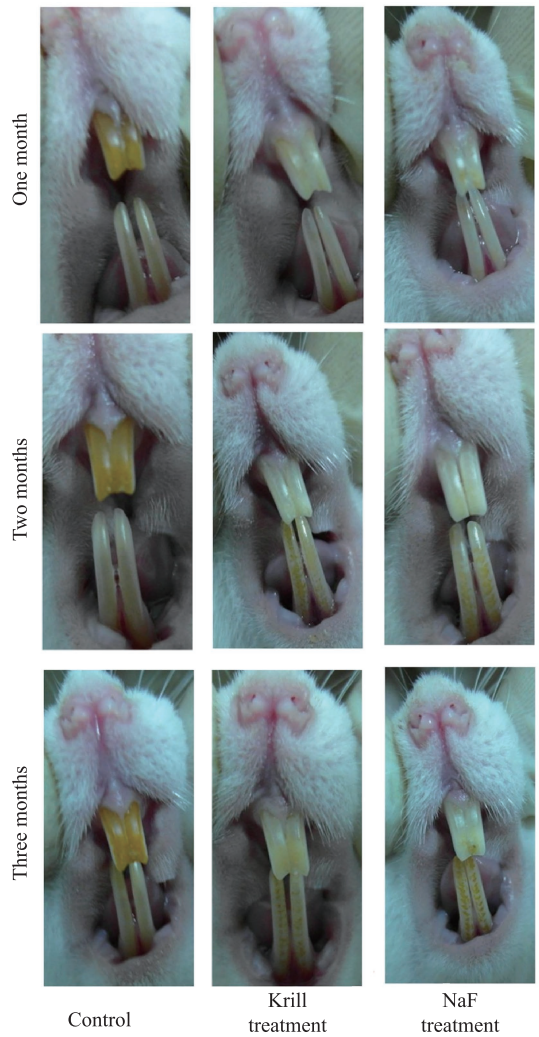

Figure 4 Photographs of the teeth of rats in the control, krill treatment, and NaF treatment groups.

The surface of the teeth of the rat in the control group was uniformly covered with pale yellow dental plaque. The dental plaque was a biofilm formed by colonizing bacteria in the oral cavity attaching to the tooth surface[26], and it was soft enough to scrape off with a fingernail. In contrast, there was no evidence of dental plaque on the teeth of the rats from the two treatment groups because F affects the metabolism of bacteria in the oral cavity and prevents the formation of dental plaque[27]. However, continued exposure to a high concentration of F may reduce the mineral content of enamel and may cause increased porosity[28]. Soft tissues do not accumulate high levels of F over time, but teeth and the skeletal system are the first affected by chronic exposure[29-30]. In the present study, the teeth of the rats from the two treatment groups displayed different degrees of white spots, brown stains, and pitting compared with the teeth of the rat from the control group. This difference is most likely related to excessive F intake in the rats from the two treatment groups.

As discussed above, the F in krill can cause dental fluorosis in rats, although the degree of dental fluorosis in the rat from the krill treatment group was less marked than in the rat from the NaF treatment group. There was an impact on aesthetics of the teeth in the rat from the krill treatment group, although the toxicity of F was less than in the rat from the NaF treatment group.

As well as dental fluorosis, high levels of exposure to F can lead to skeletal fluorosis[31-32]. Alterations in bone density and bone microstructure associated with F intake can be detected on radiographs. The 3-D bone analysis of the NaF and krill treatment groups in this study showed no significant change (P>0.05) in femur microstructure parameters (BMD, TV, BV, TS, BS, and IS) and selected trabecular bone parameters (TBPf, TbTh, TbN, and TbSp) (Tables 1 and 2). In particular, the BMD ($g \cdot L^{-1}$) of distal femur cortical bone was similar between the krill treatment group and the control group, but slightly lower than in the NaF treatment group (NS, P>0.05). Similarly, Antarctic krill-treated yellowtail had a significantly increased level of F in vertebral bones, but F in Antarctic krill did not adversely affect the growth of yellowtail[14].

The possible relationship between F intake and the risk of fractures has been extensively investigated, but results have been inclusive with some studies demonstrating deleterious effects[33-34] and some demonstrating no effect[35-36]. In this study, we did not find any significant changes in femur microstructure, possibly because the rats in the treatment groups were only exposed to increased F levels for 90 days, which may not have been long enough for the development of significant changes.

3.4 Possible mechanisms for lower toxicity of F in Antarctic krill

The results of our previous study[37] on pathological changes in soft tissues (liver, kidney, spleen, and brain) in rats fed with krill, and the results of the present study indicated that F in krill is toxic to rats. However, the F in krill was less toxic to rats than the F in NaF. This finding could be explained by two possible mechanisms. The F in krill may exist in a less toxic form and some other substances in krill may play an important role in reducing the toxicity of F.

The concentration of F in feed and feces was similar in the krill and NaF treatment groups, but the plasma inorganic

Table 1 Micro-CT analysis of distal femur cancellous bone

	Control	Krill treatment	NaF treatment
BMD/(g·L^{-1})	0.316 3±0.067 1	0.364 1±0.078 5	0.348 4±0.091 0
TV/mm^3	43.98±9.05	39.26±7.79	45.62±6.71
BV/mm^3	3.23±1.40	3.98±1.64	4.20±2.37
BV/TV	7.64±4.12	10.79±5.60	9.89±7.19
TS/mm^2	90.83±14.37	81.62±15.80	93.53±12.61
BS/mm^2	179.63±77.71	244.20±104.75	237.60±141.85
i.s./mm^2	11.44±3.06	13.30±1.98	15.42±2.98
BS/BV	55.75±1.74	60.81±7.32	55.93±1.73
BS/TV	4.27±2.36	6.67±3.56	5.61±4.26
TB.Pf /mm^{-1}	11.67±4.72	12.05±3.57	10.58±5.13
Tb.Th/μm	0.100 5±0.014 2	0.090 1±0.016 1	0.102 3±0.014 9
Tb.N/mm^{-1}	0.804 6±0.545 8	1.278 1±0.753 1	1.057 0±0.932 7
Tb.Sp/μm	0.807 0±0.417 1	0.597 6±0.525 4	0.671 1±0.347 8

Table 2 Micro-CT analysis of distal femur cortical bone

	Control	Krill treatment	NaF treatment
BMD/(kg·mm^{-3})	0.474 7±0.079 5	0.431 1±0.092 9	0.364 5±0.030 6
TV/mm^3	33.07±8.27	35.24±4.25	41.04±3.25
BV/TV	33.89±6.10	30.20±4.58	25.17±2.82
TS/mm^2	102.66±20.54	86.33±6.03	104.54±11.78
BS/mm^2	61.50±6.31	62.78±3.06	61.36±1.50
i.s./mm^2	11.85±0.909	11.51±0.56	11.28±0.61
BS/BV	5.64±0.27	5.97±0.16	5.98±0.25
BS/TV	1.902±0.261	1.807±0.317	1.503±0.147

F in the krill treatment group was lower than in the NaF treatment group. This may be because the F absorbed from the krill diet was in organic form or other substances in krill may increase the excretion of F in the urine[38]. Chitin is found in the exoskeleton of krill and its composition in whole krill is 2.4%–2.7% DW[39]. In our previous study[17] chitin was shown to absorb inorganic F and to improve bone histomorphometry. The toxicity of F in krill may therefore be reduced by chitin and while the shelling of krill may decrease the F content it may also remove a compound that can potentially reduce the toxicity of F in krill. In our latest study[40] the F removal rate and the F adsorption capacity of calcium phosphate were higher than for chitin. Therefore, calcium phosphate may also play an important role in reducing the toxicity of F in Antarctic krill.

Some trace elements, including selenium and zinc, are present in high concentrations in krill. The concentration of selenium in Antarctic krill is 2.48–4.15 mg·kg^{-1}, 2–5 times higher than in the Chinese shrimp (Fenneropenaeus chinensis) [41]. Some studies have found that high levels of selenium increase the antioxidant capacity of blood[42], while high levels of F could reduce antioxidant capacity. The concentration of zinc in krill is 153.9±5.7 mg·kg^{-1}, 1.5 times higher than in Fenneropenaeus chinensis[41]. Zinc is an essential microelement and an indispensable component of more than 300 enzymes that play an important role in human health[43]. Meral et al.[44] and Chen et al.[45] found a significant decrease in the concentration of zinc in fluorosis patients. Bennis et al.[46] showed that zinc may decrease the absorption of F from the gastrointestinal tract. The high content of selenium and zinc in krill may therefore play an important role in decreasing the toxicity of F in krill.

4 Conclusions

The results of the present study showed that concentrations of F in plasma and bone samples from rats varied between Antarctic krill and NaF treatment groups, but F concentrations in samples from both treatment groups were significantly higher than in samples from rats in the control group. The degree of dental fluorosis in both treatment groups increased with time, but this 3-month study was probably not long enough to demonstrate significant effects on the microstructure of femur bone and trabecular bone parameters. Our findings demonstrated that F in Antarctic krill was less toxic than F in NaF, but it was still toxic to rats. The content of F in Antarctic krill needs to be addressed if krill is to be used as a food source for humans.

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